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Comparison of the mode of action of *ras* p21 with those of protein kinases A and C in the stimulation of gene expression in NIH/3T3 cells

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To compare the mode of action of *ras* p21 with those of protein kinases A and C in the regulation of gene expression in NIH/3T3 cells, we investigated the transcriptional activity of various enhancer/promoters and enhancer motifs in the cells transfected with the c-Ha-*ras*^{val12} complementary DNA (cDNA). The results indicate that the c-Ha-*ras*^{val12} protein stimulates the enhancer/promoters of the c-*fos* gene, the metallothionein II_A gene, the simian virus 40 (SV40) virus genome and the Rous sarcoma (RS) virus genome, and the serum-response element and the 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-response element in a manner independent of protein kinases A and C in NIH/3T3 cells.

ras p21; Protein kinases A and C; Enhancer/promoter; NIH/3T3 cell

1. INTRODUCTION

The *ras* p21 and its activated form are involved in the proliferation of various types of cells (for a review, see [1]). It has been reported that *ras* p21 may regulate the phospholipase C-mediated hydrolysis of phosphoinositides and consequently modulate protein kinase C activity in mammalian cells such as NIH/3T3 cells [2,3], but the direct linkage between the phospholipase C and *ras* p21 has not yet been obtained.

On the other hand, the activated *ras* p21 stimulates the enhancer/promoters of the c-*fos* gene and the polyoma virus genome [4–6]. The enhancer/promoters of the c-*fos* gene and the polyoma virus genome contain the SRE[2], the CRE and/or the TRE, and the transcriptional activities of these enhancer/promoters are regulated by these elements [7–10]. The SRE and the TRE are activated by TPA though protein kinase C in various types of cells including NIH/3T3 cells [8–12].

In the present studies, we have examined the relationship between the action of the activated *ras* p21 and those of protein kinases A and C in the stimulation of various enhancer/promoters and enhancer motifs in NIH/3T3 cells. This paper shows that the activated *ras* p21 stimulates the enhancer/promoters of the c-*fos* gene, the metallothionein II_A gene, the SV40 virus genome and the RS virus genome, and the SRE and the TRE in a manner independent of protein kinases A and C in NIH/3T3 cells.

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Abbreviations: SRE, serum-response element; CRE, cyclic AMP-response element; TRE, 12-*O*-tetradecanoylphorbol-13-acetate-response element; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; protein kinase A, cyclic AMP-dependent protein kinase; SV40, simian virus 40; RS, Rous sarcoma; CAT, chloramphenicol acetyltransferase; cDNA, complementary DNA; Bt_2cAMP, dibutyryl cyclic AMP; PDBu, phorbol-12,13-dibutyrate; PDGF, platelet-derived growth factor; NGF, nerve growth factor

2. MATERIALS AND METHODS

2.1. *Materials and chemicals*

NIH/3T3 cells were donated by M. Wigler (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY). The pcDSRα expression plasmid, pSV2CAT, pRSVCAT, c-*fos*-luciferase and pRSV-luciferase were donated from K. Arai (DNAX Research Institute, Palo Alto, CA) [13–16]. c-*fos*CAT (FC4) was a gift from I.M. Verma (Salk Institute, San Diego, CA) [17]. pMTCAT (phMTCAT-4) was from K. Nose (Tokyo University, Tokyo, Japan) [18]. Somatostatin-CAT (pR1-CAT) was from R.H. Goodman (Tufts-New England Medical Center, Boston, MA) [19]. pTKGH was purchased from Nicols Institute [20].

2.2. *Construction of plasmids*

pcDSRα*ras* and pcDSRα*ras*^{val12} were constructed for expression of normal (Gly-12) and activated (Val-12) human c-Ha-*ras* p21, respectively, as described [6]. c-*fos*CAT and c-*fos*-luciferase contain the 0.4-kilobase 5′-flanking sequence of the c-*fos* gene upstream from the CAT and luciferase genes, respectively [16,17]. pSV2CAT contains the 0.6-kilobase enhancer/promoter region of the SV40 virus genome upstream from the CAT gene [14]. pRSVCAT and pRSV-luciferase contain the 0.5-kilobase enhancer/promoter region of the RS virus genome upstream from the CAT and luciferase genes, respectively [15,16]. pMTCAT and somatostatin-CAT have the 0.4-kilobase 5′-flanking sequence of the human metallothionein II_A and the 4-kilobase 5′-flanking sequence of the somatostatin genes, respectively, upstream from the CAT genes [18,19]. ΔIL3CAT was constructed as described [21]. SRECAT, CRECAT and TRECAT were constructed as described [12].

2.3. *Assays for CAT, growth hormone and luciferase*

NIH/3T3 cells were transfected with 20 μg of plasmid DNA, con-

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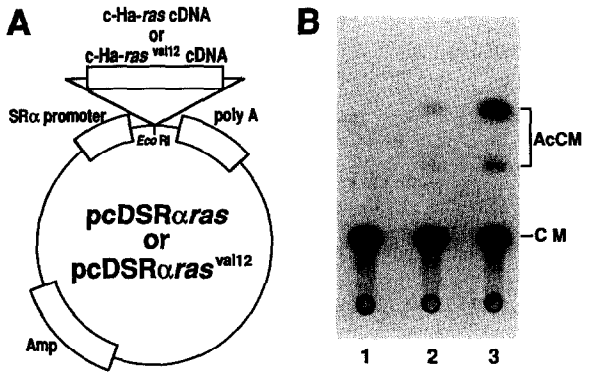

Fig. 1. Activation of c-*fos*CAT by cotransfection of the c-Ha-*ras*^{val12} cDNA to NIH/3T3 cells. (A) Construction of expression plasmids harboring the c-Ha-*ras* cDNAs. The c-Ha-*ras* and c-Ha-*ras*^{val12} cDNAs were cloned into the pcDSRα expression plasmid to yield pcDSRα*ras* and pcDSRα*ras*^{val12} as described [6]. (B) c-*fos* CAT expression. pcDSRα*ras* or pcDSRα*ras*^{val12} was cotransfected with c-*fos*CAT and pTKGH to NIH/3T3 cells. After 48 h incubation, CAT activity and the levels of growth hormone were assayed as described under section 2. Lane 1, control; lane 2, pcDSRα*ras*; lane 3, pcDSRα*ras*^{val12}. CM and AcCM represent chloramphenicol and its acetylated forms, respectively.

taining 5 μg of pcDSRα*ras*^{val12}, 5 μg of pTKGH, 5 μg of the CAT construct or 5 μg of the luciferase construct, and pUC18 as a carrier as indicated. The levels of the human growth hormone were measured as described [12]. CAT and luciferase activities were measured as described [14,16].

3. RESULTS

3.1. *Expression of the CAT and luciferase fusion genes by cotransfection of the c-Ha-*ras^{val12} *cDNA*

The c-Ha-*ras* and c-Ha-*ras*^{val12} cDNAs were cloned into the pcDSRα expression plasmid under the control of the SRα promoter (pcDSRα*ras* and pcDSRα*ras*^{val12}) (Fig. 1A) [6]. pcDSRα*ras*^{val12} showed transforming activity after transfection to NIH/3T3 cells (data not shown). Various CAT fusion genes (c-*fos*CAT, pMTCAT, pSV2CAT, pRSVCAT and somatostatin-CAT) were tested for their responses to the *ras* constructs. To measure the relative efficiency of transfection and to normalize the separate experiments, the human growth hormone gene under the transcriptional control of the promoter of the thymidine kinase gene (pTKGH) was cotransfected with the CAT fusion genes to NIH/3T3 cells. Cotransfection of pcDSRα*ras*^{val12} or addition of TPA directed expression of c-*fos*CAT, pMTCAT, pSV2CAT and pRSVCAT but not somatostatin-CAT (Fig. 1B and Table I). Addition of Bt_2cAMP directed expression of c-*fos*CAT, pSV2CAT, pRSVCAT and somatostatin-CAT but not pMTCAT. Cotransfection of pcDSRα*ras* conferred a minimal increment of CAT activity derived from these CAT fusion genes. Cotransfection of the control plasmid (pcDSRα) alone showed no effect on CAT expression. CAT activity derived from the CAT construct lacking the enhancer/promoter sequence was negligible in the cells cotransfected with the *ras* constructs or supplied with TPA or Bt_2cAMP (data not shown).

To examine the reporter gene other than the CAT gene, the enhancer/promoter of the c-*fos* gene or the RS virus genome ligated to the luciferase gene (c-*fos*-luciferase or pRSV-luciferase) was employed as another reporter of transcriptional activity of the enhancer/promoter of the c-*fos* gene or the RS virus genome. c-*fos*-luciferase or pRSV-luciferase and pTKGH were transfected to NIH/3T3 cells. Cotransfection of pcDSRα*ras*^{val12} or addition of TPA or Bt_2cAMP to NIH/3T3 cells directed expression of c-*fos*-luciferase and pRSV-luciferase (Table II). Since the luciferase fusion genes contain the signal for a polyadenylation addition upstream from the enhancer/promoters, the background level of the expression was very low compared with those of the CAT fusion genes [16]. The levels of the growth hormone derived from pTKGH were almost the same among the transfected cells in each experiment (data not shown).

3.2. *SRECAT and TRECAT expression by cotransfection of the c-Ha-*ras^{val12} *cDNA*

The enhancer/promoter sequences of the CAT fusion genes described above contain the various

Table I

Activation of the CAT fusion genes by cotransfection of the c-Ha-*ras*^{val12} cDNA to NIH/3T3 cells

	c-*fos*CAT	pMTCAT	pSV2CAT	pRSVCAT	Somatostatin-CAT
None	1.5% (1.0)	2.4% (1.0)	2.0% (1.0)	4.6% (1.0)	0.7% (1.0)
TPA (100 nM)	6.8% (4.5)	5.0% (2.1)	4.5% (2.3)	12.0% (2.6)	0.9% (1.3)
Bt_2cAMP (2 mM)	4.3% (2.9)	2.7% (1.1)	4.4% (2.2)	10.6% (2.3)	7.2% (10.0)
pcDSRα	1.5% (1.0)	2.3% (1.0)	2.0% (1.0)	4.9% (1.1)	0.8% (1.1)
pcDSRα*ras*	2.1% (1.3)	2.4% (1.0)	2.5% (1.3)	6.1% (1.3)	0.7% (1.0)
pcDSRα*ras*^{val12}	7.5% (5.0)	8.4% (3.5)	6.7% (3.4)	37.0% (8.0)	0.9% (1.3)

pcDSRα, pcDSRα*ras* or pcDSRα*ras*^{val12} was cotransfected with c-*fos*CAT, pMTCAT, pSV2CAT, pRSVCAT or somatostatin-CAT and pTKGH to NIH/3T3 cells. TPA or Bt_2cAMP was added to the cells transfected with the CAT fusion genes and pTKGH 8 h before the harvest of the cells. CAT activity and the levels of growth hormone were then assayed as described under section 2. Each value represents the percentage of the conversion of chloramphenicol into the acetylated forms. The numbers in parentheses indicate the fold of induction. Essentially identical results were obtained in three independent experiments

Table II

Activation of the luciferase fusion genes by cotransfection of the c-Ha-*ras*^{val12} cDNA to NIH/3T3 cells

	c-*fos*-luciferase	pRSV-luciferase
	(relative light units)	
None	2	2
TPA (100 nM)	83	124
Bt_2cAMP (2 mM)	62	92
pcDSRα	2	2
pcDSRα*ras*	20	31
pcDSRα*ras*^{val12}	134	422

pcDSRα, pcDSRα*ras* or pcDSRα*ras*^{val12} was cotransfected with c-*fos*-luciferase or pRSV-luciferase and pTKGH to NIH/3T3 cells. TPA or Bt_2cAMP was added to the cells transfected with the c-*fos*-luciferase or pRSV-luciferase and pTKGH 8 h before the harvest of the cells. Luciferase activity and the levels of growth hormone were then assayed as described under section 2. Essentially identical results were obtained in three independent experiments

Table III

Activation of SRECAT and TRECAT by cotransfection of the c-Ha-*ras*^{val12} cDNA to NIH/3T3 cells

	SRECAT	CRECAT	TRECAT
None	0.5% (1.0)	0.6% (1.0)	1.1% (1.0)
TPA (100 nM)	2.0% (4.0)	0.6% (1.0)	3.9% (3.5)
Bt_2cAMP (2 mM)	0.6% (1.2)	4.6% (7.7)	1.3% (1.2)
pcDSRα	0.5% (1.0)	0.7% (1.1)	1.2% (1.1)
pcDSRα*ras*	0.8% (1.6)	0.7% (1.1)	1.9% (1.7)
pcDSRα*ras*^{val12}	2.9% (5.8)	0.8% (1.3)	6.6% (6.0)

pcDSRα, pcDSRα*ras* or pcDSRα*ras*^{val12} was cotransfected with SRECAT, CRECAT or TRECAT and pTKGH to NIH/3T3 cells. TPA or Bt_2cAMP was added to the cells transfected with the CAT fusion genes and pTKGH 8 h before the harvest of the cells. CAT activity and the levels of growth hormone were then assayed as described under section 2. Each value represents the percentage of the conversion of chloramphenicol into the acetylated forms. The numbers in parentheses indicate the fold of induction. Essentially identical results were obtained in three independent experiments

enhancer motifs such as the SRE, the CRE and/or the TRE [7–12]. To determine which element is responsible for the *ras*^{val12} p21-induced expression of the CAT fusion genes, the SRE, the CRE and the TRE were synthesized and inserted into ΔIL3CAT, which lacked the enhancer motif but contained the intact mouse interleukin 3 promoter region, to yield SRECAT, CRECAT and TRECAT, respectively [12]. ΔIL3CAT consists of the transcriptional initiation site and the CAT reporter gene [21]. Cotransfection of pcDSRα*ras*^{val12} with SRECAT or TRECAT to NIH/3T3 cells increased CAT activity, whereas cotransfection with CRECAT did not increase CAT activity (Table III). Similar results were obtained when the cells transfected with these CAT fusion genes were stimulated by TPA. Bt_2cAMP increased CAT activity in the cells transfected with CRECAT but not with SRECAT or TRECAT. Neither cotransfection of pcDSRα*ras*^{val12} nor the addition of TPA or Bt_2cAMP to the cells transfected with ΔIL3CAT increased CAT activity (data not shown).

3.3. *Effect of the down-regulation of protein kinase C on SRECAT, CRECAT and TRECAT expression*

In the last set of experiments, it was examined whether the *ras*^{val12} p21-induced expression of the CAT fusion genes is mediated through protein kinase C. Prolonged treatment of NIH/3T3 cells with PDBu caused the down-regulation of protein kinase C as described [12]. In these cells, TPA did not stimulate SRECAT or TRECAT expression, whereas transfection of pcDSRα*ras*^{val12} induced expression of these CAT fusion genes in the control and PDBu-treated cells to a similar extent (Table IV). Bt_2cAMP also induced CRECAT expression in both the control and PDBu-treated cells (data not shown).

Table IV

Activation of SRECAT and TRECAT by cotransfection of the c-Ha-*ras*^{val12} cDNA to the PDBu-treated NIH/3T3 cells

	SRECAT		TRECAT	
	Control cells	PDBu-treated cells	Control cells	PDBu-treated cells
None	0.5% (1.0)	0.6% (1.0)	1.0% (1.0)	0.9% (1.0)
TPA (100 nM)	2.1% (4.2)	0.6% (1.0)	3.7% (3.7)	0.9% (1.0)
pcDSRα*ras*^{val12}	3.2% (6.4)	3.3% (5.5)	6.3% (6.3)	6.1% (6.8)

pcDSRα*ras*^{val12} was cotransfected with SRECAT or TRECAT and pTKGH to NIH/3T3 cells. The cells were incubated with 800 nM PDBu for 40 h. After washing with phosphate-buffered saline, TPA was added to the cells transfected with the CAT fusion genes 8 h before the harvest of the cells. CAT activity was measured as described under section 2. Each value represents the percentage of the conversion of chloramphenicol into the acetylated forms. The numbers in parentheses indicate the fold of induction. Essentially identical results were obtained in three independent experiments

4. DISCUSSION

We have first shown here that the activated *ras* p21 stimulates the enhancer/promoters of the c-*fos* gene, the metallothionein II_A gene, the SV40 virus genome and the RS virus genome, and the SRE and the TRE in NIH/3T3 cells. We have then shown here that TPA stimulates these enhancer/promoters and enhancer motifs, but that Bt_2cAMP stimulates the enhancer/promoters of the c-*fos* and the somatostatin genes, the SV40 virus genome and the RS virus genome, and the CRE in NIH/3T3 cells. Thus, the specificity of the activated *ras* p21 towards these enhancer/promoters and enhancer motifs is similar to that of TPA but different from that of Bt_2cAMP. It is likely from this result that the activated *ras* p21 and protein kinase C stimulate these enhancer/promoters and enhancer motifs in a manner independent of protein kinase A in NIH/3T3 cells. We have also shown here that the activated *ras* p21 stimulates the SRE and the TRE even in the cells whose protein kinase C is down-regulated to the same extent as those induced in the control cells. In these protein kinase C-down-regulated cells, TPA does not activate these elements. These results suggest that the activated *ras* p21 stimulates these enhancer motifs in a manner independent of protein kinase C in NIH/3T3 cells.

We have previously shown that the activated *ras* p21 activates the c-*fos* gene enhancer/promoter and the SRE but not the CRE in a manner independent of protein kinases A and C in PC12 cells [6]. PC12 cells are differentiated into neuron-like cells in response to NGF and Bt_2cAMP [22,23]. The activated *ras* p21 induces the differentiation of PC12 cells and the microinjection of an anti-*ras* p21 monoclonal antibody inhibits the NGF-induced differentiation of PC12 cells [24,25]. NGF activates the c-*fos* gene enhancer/promoter and the SRE but not the CRE in a manner independent of protein kinases A and C [6]. It is likely from these observations that *ras* p21 is involved in the NGF-induced differentiation of PC12 cells into neuron-like cells. The present results together with these earlier observations indicate that *ras* p21 induces expression of genes having at least the SRE and the TRE in a manner independent of protein kinases A and C in both proliferating and differentiating states of cells.

It has been considered that *ras* p21 serves as a transducer for extracellular signals such as growth factors [1,25–28]. The extracellular signals for the activation of *ras* p21 have not, however, been determined. In this regard, it has recently been shown that PDGF receptor phosphorylates GTPase activating protein for *ras* p21 (*ras* GAP) and may be linked to the regulation of *ras* p21 activity in NIH/3T3 cells [28–30]. PDGF is known to stimulate the c-*fos* gene enhancer/promoter and the SRE [31]. Thus, it could be speculated that PDGF induces gene expression through the activation of *ras* p21. It has been also shown that expression of the activated *ras* p21 stimulates the phosphorylation of the c-*raf* protein and may consequently activate its protein kinase activity [32,33]. We have recently shown that transfection of the cDNA of the activated c-*raf* protein stimulates the c-*fos* gene enhancer/promoter, the SRE and the TRE in NIH/3T3 cells [12]. A similar observation has been made by Jamal and Ziff [34]. Therefore, it is tempting to speculate that *ras* p21 induces gene expression through the activation of the c-*raf* protein in NIH/3T3 cells. Further studies are necessary to understand the modes of activation and action of *ras* p21 and its physiological role in the regulation of gene expression for growth and differentiation control.

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